

Activity of the A3 adenosine receptor gene promoter in transgenic mice: characterization of previously unidentified sites of expression

Ron Yaar, Edward D. Lamperti, Paul A. Toselli, Katya Ravid*

Department of Biochemistry and Whitaker Cardiovascular Institute, Boston University School of Medicine, K225, 715 Albany Street, Boston, MA 02118, USA

Received 30 September 2002; accepted 7 October 2002

First published online 18 November 2002

Edited by Jesus Avila

Abstract Sites of A3 adenosine receptor gene expression have not been fully explored nor has this gene's promoter activity been confirmed in vivo. Transgenic mice were generated in which 2.3 kb upstream of the transcriptional start site of the mouse A3 adenosine receptor was coupled to a β -galactosidase reporter gene. Selective transgene expression was detected in testis and brain as well as at other sites in which A3 adenosine receptor message has not been previously reported, including retinal ganglion cells and smooth muscle cells of the cerebrospinal vasculature. Our study suggests that this promoter may be useful in the selective targeting of gene expression to specific tissues. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Adenosine; Purinergic P1 receptor; Nucleic acid regulatory sequence; Reporter gene

1. Introduction

Adenosine is a natural metabolite that plays a role both in normal cell physiology and in certain pathological processes. To date, four adenosine receptors – A1, A2a, A2b, and A3 – have been cloned [1]. All four are G protein-coupled receptors that have the capacity to modulate intracellular cAMP levels. Receptors for adenosine are natural candidates for investigating the mechanisms through which adenosine influences human physiology and may serve as a tool for specifically targeting adenosine activity.

The most recently cloned adenosine receptor, the A3 adenosine receptor, has been implicated in diverse processes such as cell growth [2], ischemia/reperfusion injury [3], and modulation of inflammatory responses [4,5]. There are some discrepancies in published descriptions regarding the tissue distribution of A3 adenosine receptor message in the rat and the human [6–12]. In the rat, however, there is considerable evidence for high levels of expression in the testis, with lower levels in brain, lung, and other tissues.

Transgenic mice are commonly used to analyze the expression of DNA constructs in vivo, and the bacterial *lacZ* gene

has been employed frequently to determine spatial and temporal specificity of the control of transcription by promoter elements. A 2.3 kb mouse genomic fragment 5' upstream of the coding region for the mouse A3 adenosine receptor demonstrates promoter activity in transient transfection studies [13]. We have generated transgenic mice using a reporter construct composed of this fragment driving the expression of *Escherichia coli lacZ*. These mice were analyzed for expression of *lacZ* mRNA by reverse transcription-polymerase chain reaction (RT-PCR), and for β -galactosidase activity by histochemistry. This profile of *lacZ* expression was compared to endogenous A3 adenosine receptor mRNA levels, as determined by in situ hybridization to RNA probes, and by RT-PCR. Our goal was to determine whether 2.3 kb of the mouse A3 adenosine receptor promoter could recapitulate A3 adenosine receptor expression in vivo and reveal previously unknown locations of expression.

2. Materials and methods

2.1. Generation of constructs

All restriction endonucleases were acquired from New England Biolabs (Beverly, MA, USA). The pSDKLacZpA and pA3HGH constructs have been described previously [13,14]. A *Bam*HI fragment of the pA3HGH plasmid, containing 2.3 kb of the 5' non-coding region of the mouse A3 adenosine receptor gene, was inserted upstream of the *E. coli lacZ* coding region of pSDKLacZpA. Proper orientation of the ligated fragments was confirmed by sequencing, and the resulting plasmid, termed pSA3Z, was purified by CsCl density gradient centrifugation.

The pA3INSITU plasmid was generated by cloning a 350 bp PCR product, derived from exon II of the mouse A3 adenosine receptor gene, into the pCRII vector, using the pCRII TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Primers (Invitrogen) mA3EX2-1F (5'-GTT TCC TTT CTG GTG GGG C-3') and mA3EX2-1R (5'-GCA CAT TGC GAC ATC TGG TAT C-3') were used to generate the insert. Orientation and sequence fidelity of the final construct were confirmed by sequencing and the resulting plasmid was purified by CsCl density gradient centrifugation.

2.2. Generation of transgenic mice

pSA3Z was digested with *Kpn*I and fractionated by agarose gel electrophoresis. A 5.5 kb fragment, containing the mouse A3 adenosine receptor promoter, *lacZ* coding region, and SV40 polyadenylation signal, was excised from the gel and purified by electroelution followed by ethanol precipitation. DNA was resuspended using injection buffer (7.5 mM Tris-HCl, 0.15 mM EDTA, pH 7.4). Injection of DNA into the pronuclei of fertilized eggs, derived from FVB mice, was performed at Boston University's Transgenic Core, as described previously [14,15]. Incorporation of the transgenic fragment was confirmed by Southern blotting. The probe used for Southern blotting was generated by *Eco*RI and *Eco*RV digestion of pSA3Z/*Kpn*I DNA and gel purification of a 1.9 kb fragment and was specific for the *E. coli lacZ* coding region (see Fig. 1).

*Corresponding author. Fax: (1)-617-638 5054.

E-mail address: ravid@biochem.bumc.bu.edu (K. Ravid).

Abbreviations: Bluo-gal, halogenated indolyl- β -D-galactoside; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; VSMC, vascular smooth muscle cells; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

2.3. Reverse transcription coupled to the polymerase chain reaction

Animals were anesthetized by inhalation of isoflurane and exsanguinated. Tissue was dissected and immediately rinsed in ice-cold 1×phosphate buffered saline (PBS) and homogenized in Trizol (Invitrogen) using a Virtis Handishear handheld homogenizer. RNA was purified using the Trizol RNA isolation kit as directed by the manufacturer and resuspended in 0.1% diethyl pyrocarbonate treated water (American Bioanalytical, Natick, MA, USA).

RT-PCR of tissue RNA was performed as described previously [16] with the following modifications: PCR was carried out in a total volume of 25 µl, containing a final concentration of 1×PCR buffer, 1.5 mM MgCl₂, 200 µM each dNTP, 0.5 µM each of the two primers used, and 1 U of Taq DNA Polymerase (Promega, Fitchburg, WI, USA). For amplification of glyceraldehyde phosphate dehydrogenase (GAPDH) message, 1 µl of reverse transcription was used as template, whereas 2 µl of reverse transcription was used as template for amplification of the mouse A3 adenosine receptor message and for amplification of *lacZ* message. Annealing temperatures of 55°C for GAPDH message amplification, 57°C for A3 adenosine receptor message amplification, and 59°C for *lacZ* message amplification were used. Reactions were performed with 30 cycles of amplification. All primers were acquired from Invitrogen. Primers used for A3 adenosine receptor message amplification from mouse tissue were mA31-F (5'-ACG GAC TGG CTG AAC ATC ACC TAC-3') and mA31-R (5'-TGT AAT CCA AAC TGA CCA CGG AAC-3'). Primers used for A3 adenosine receptor message amplification from rat tissue were RATA3-F (5'-TTG CTG TAG ACC GAT ACC TGC GAG-3') and RATA3-R (5'-TGA ACT CCC GAC CGT AAA ATG C-3'). Primers used for *lacZ* message amplification were LACZ1-F (5'-CTA TCG TGC GGT GGT TGA ACT G-3') and LACZ1-R (5'-CCA GGT AGC GAA AGC CAT TTT TTG-3'). RT-PCR products were separated on a 1.2% agarose gel containing ethidium bromide and visualized under UV light.

2.4. Preparation of mice for β-galactosidase assay and in situ hybridization

Mice were anesthetized by inhalation of ether and perfused through a needle in the left ventricle. Mice were perfused with 6 ml of 1×PBS followed immediately by 24 ml of 2% formaldehyde or a mixture of 4% formaldehyde/0.25% glutaraldehyde (American Bioanalytical) at a rate of 6 ml/min using a peristaltic pump. Perfusion of fixative was continued for 15 min at 2.75 ml/min, followed by perfusion of 1×PBS for 10 min. Mice prepared for in situ hybridization analysis were perfused with 6 ml of 1×PBS followed immediately with 24 ml of 4% formaldehyde at a rate of 6 ml/min. Perfusion of fixative was then continued for 25 min at 2.75 ml/min.

2.5. β-Galactosidase assay and histology

Tissue was dissected from mice and stored in ice-cold 1×PBS prior to staining for β-galactosidase activity. Individual organs were cut into 0.5–1 mm thick slices, and stained as described previously [17]. The staining solution contained a final concentration of 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) or halogenated indolyl-β-D-galactoside (Bluo-gal; Invitrogen). Samples were incubated at 37°C for 6–12 h on a rocking platform. All locations of specific β-galactosidase activity were detected within 6 h of staining.

Tissue used for paraffin and plastic histology was dehydrated in a graded ethanol series. Samples destined for paraffin sectioning were cleared with xylenes and embedded with paraffin in a heated vacuum oven. Paraffin blocks were stored at 4°C and cut at a thickness of 5 µm. Slides for in situ analysis were stored at 4°C for less than 1 month. Tissue for electron microscopy was prepared essentially as described previously, except that staining with contrast reagents was specifically omitted [18].

2.6. In situ hybridization

The pA3INSITU plasmid was linearized with the restriction enzymes *EcoRV* and *HindIII*. ³⁵S-labeled sense and antisense RNA was generated from *EcoRV*- and *HindIII*-digested plasmid, respectively. SP6 RNA polymerase (Promega) was used to generate sense strands, while T7 RNA polymerase (Promega) was used to generate antisense strands. The expected probe size was 350 bases. In situ hybridization was performed as described previously [15].

3. Results

3.1. Generation of pSA3Z mice

In order to examine promoter activity in vivo, transgenic mice (pSA3Z) were generated using a transgenic construct containing 2.3 kb of the 5' flanking region of the mouse A3 adenosine receptor gene coupled to *E. coli lacZ* (Fig. 1). Four independent founders were identified by Southern blot analysis of genomic DNA. Three founder lines (lines #5, #26, and #33) incorporated a similar number of transgene copies, while the fourth (line #29) incorporated a significantly greater number of transgene copies. pSA3Z mice were not visibly distinguishable from wild type littermates, bred normally, and transmitted the transgene in a Mendelian fashion. RT-PCR analysis of RNA derived from testis, brain, spleen, kidney, heart, lung, uterus, and skeletal muscle of mice from each transgenic line showed no significant changes in endogenous A3 adenosine receptor mRNA level compared to wild type controls (data not shown).

3.2. Distribution of A3 adenosine receptor message in mouse testis and β-galactosidase activity in testis and retina of transgenic mice

Testis is the most abundant source of A3 adenosine receptor mRNA in the mouse. We used the testis of our transgenic pSA3Z mice for a detailed comparison of the profile of β-galactosidase activity, driven by the A3 receptor promoter, with the pattern of endogenous A3 adenosine receptor mRNA

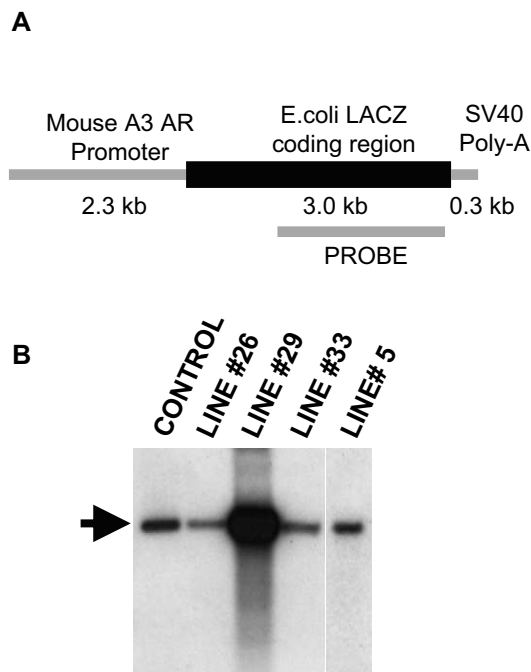


Fig. 1. Generation of transgenic mice carrying pSA3Z. A: Schematic of the *KpnI* digestion of pSA3Z showing the relative position and size of the *Bam*HI fragment of the mouse A3 adenosine receptor promoter, the *E. coli lacZ* coding region, and the SV40 polyA signal. The region corresponding to the probe used for Southern analysis is indicated. B: Southern analysis of mouse tail DNA, digested with *EcoRI* and *EcoRV*, from offspring of transgenic mice of the four independent lines generated. Approximately 10 µg of genomic DNA was loaded in each lane; the control lane contained 50 pg of an *EcoRI/EcoRV* digestion of the construct used for generating the mice. The arrow points to the expected 1.9 kb product.

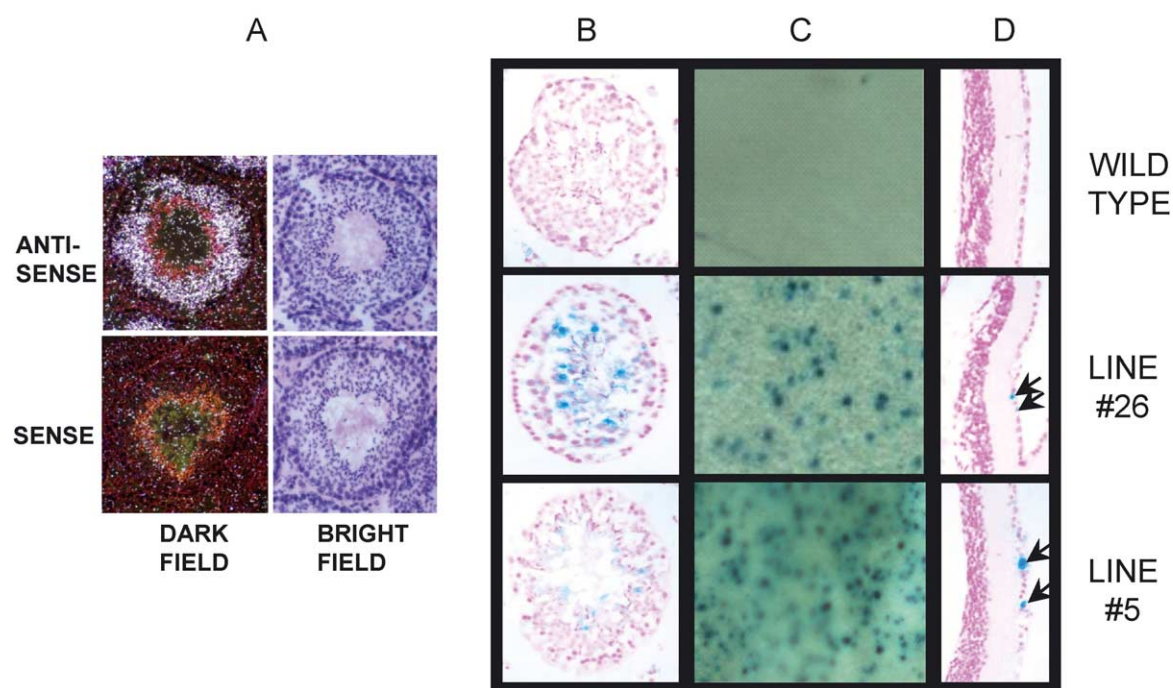


Fig. 2. Endogenous A3 adenosine receptor message and β -galactosidase activity in testis and retina of transgenic mice. A: 16 μ m cryosections of wild type mouse testis were subjected to in situ hybridization using a radiolabeled sense or antisense probe derived from the second exon of the mouse A3 adenosine receptor gene. Sections were lightly stained with hematoxylin and eosin and visualized for specific hybridization of probe. B: Testes from wild type, #26, and #5 line mice were stained 6 h for β -galactosidase activity. 5 μ m paraffin sections, counterstained with nuclear fast red, are shown. C,D: Eyes from wild type, #26, and #5 line mice were dissected and stained 6 h for β -galactosidase activity. C: After staining, retina was dissected and small segments examined in whole-mount preparations at 40 \times magnification. D: 5 μ m paraffin cross-sections of retina, counterstained with nuclear fast red, are shown. Arrows indicate locations of β -galactosidase activity.

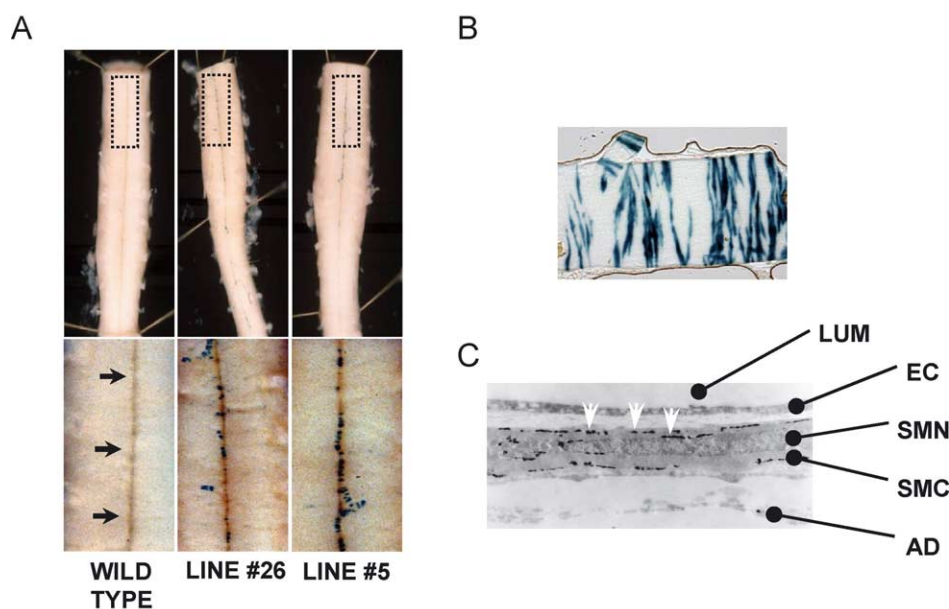


Fig. 3. β -Galactosidase activity in the cerebrospinal vasculature of transgenic mice. Spinal cord and brain of wild type, #26, and #5 line mice were stained for β -galactosidase activity with X-gal (A,B) or BluO-gal (C). A: Ventral aspect of the caudal spinal cord of wild type, #26, and #5 line mice. The boxed area is magnified in the lower panel. Arrows indicate the location of the anterior spinal artery in control mice. B: Whole mount of the basilar artery of a #26 line transgenic mouse at 40 \times magnification. C: Electron microscopy of plastic sections of a #26 line anterior spinal artery stained for β -galactosidase activity. Arrows indicate location of deposits of β -galactosidase product. LUM, lumen of artery; EC, endothelial cell; SMN, smooth muscle cell nucleus; SMC, smooth muscle cell cytoplasm; AD, adventitia.

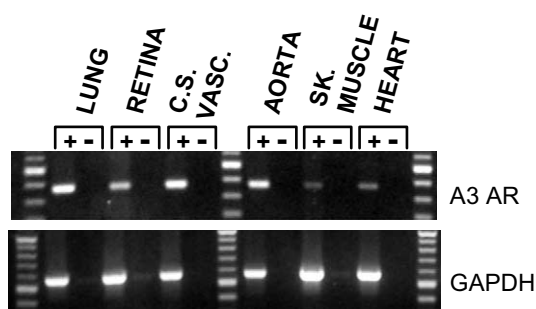


Fig. 4. Endogenous A3 adenosine receptor message in cerebrospinal vasculature and retina. RNA generated from tissues of female Sprague–Dawley rats was reverse-transcribed (+ and – indicate the presence or absence of reverse transcriptase during the incubation). PCR was subsequently performed with primers specific for endogenous rat A3 adenosine receptor message and for GAPDH message.

expression. We found that endogenous A3 mRNA was highly expressed and widely distributed within the seminiferous tubules (Fig. 2A). This distribution is consistent with expression of A3 adenosine receptor mRNA in developing germ cells. A3 mRNA was not detected in the periphery or lumen of the tubule, or in other parts of the testis.

Testes of wild type and pSA3Z mice were analyzed for expression of the *lacZ* gene product by RT-PCR, using transgene-specific primers, and histochemically by incubation with X-gal, a substrate which is converted ultimately into a deep blue precipitate by β -galactosidase activity. RT-PCR indicated transgene expression in the testis in all four lines (not shown). Gross inspection of histochemical preparations revealed specific β -galactosidase activity in the testis in two of the transgenic lines (#26 > #5); high background staining may have obscured lower-level transgene expression in the other two lines. As described below, histochemical staining was generally more intense for the #26 line in all of the tissues where the transgene was active. As expected from various other studies,

the level of transgene expression did not correlate with transgene copy number in each line. Testicular β -galactosidase activity was distributed in a periluminal fashion, with increasing intensity towards the center of the seminiferous tubule (Fig. 2B). No specific β -galactosidase activity was detected at the periphery of the seminiferous tubule or in other parts of the testis.

Analysis of different organs revealed specific β -galactosidase activity in the retina in three transgenic lines (#5, #26, and #29). β -Galactosidase staining was localized to a subset of retinal ganglion cells that was evenly distributed within the retina (Fig. 2C,D).

3.3. β -Galactosidase activity in cerebrospinal vasculature of transgenic mice

Specific β -galactosidase activity was detected in the cerebrospinal vasculature of two transgenic lines (Fig. 3A). A segment of basilar artery was stained for β -galactosidase activity and examined at low magnification, under a light microscope. The profile of β -galactosidase staining activity in this artery was consistent with that of vascular smooth muscle cells (VSMC), although only a subset of these cells was stained (Fig. 3B). Specific staining was also observed in the anterior spinal artery, the vasculature on the ventral surface of the cerebral hemispheres and brainstem, and in vasculature within the brainstem (Fig. 3A,B, and data not shown). To confirm that cells staining for β -galactosidase activity were, in fact, VSMC, we stained anterior spinal artery of mice of line #26 with Bluo-gal, a substrate for β -galactosidase that generates an electron-dense precipitate. Examination at the electron microscopic level demonstrated specific deposition of precipitate in a subset of VSMC (Fig. 3C). No precipitate could be detected in the adventitial or endothelial cell layers of the vessel. No specific β -galactosidase activity could be observed histochemically in other vessels, including aorta, carotid arteries, and iliac arteries (data not shown).

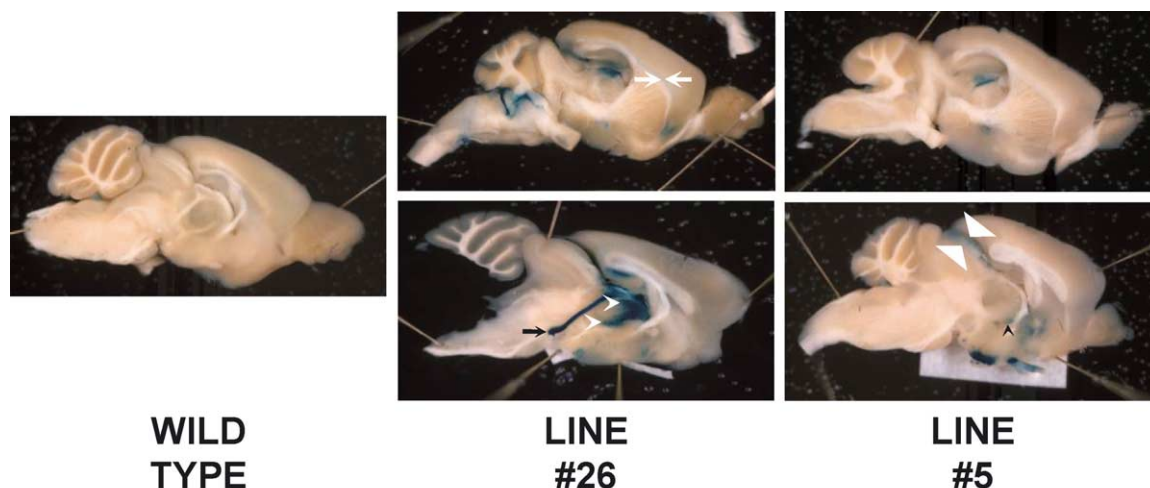


Fig. 5. β -Galactosidase activity in brains of transgenic mice. Sagittal brain slices from wild type, #26, and #5 line mice were stained for β -galactosidase activity. Shown are two representative sections each, at different levels, from #26 and #5 line mice. In the upper panel for the #26 line, the white arrows demarcate the position of scattered β -galactosidase-positive neurons in the cerebral cortex. In the lower panel, the black arrow marks the position of the interpeduncular nucleus, contiguous with the similarly positive habenulo-interpeduncular tract, which appears as a dark blue diagonal stripe. The white arrowheads show the position of thalamic staining. In the lower panel for the #5 line, the white triangles show the position of the superior colliculus, light-blue stained, and the black arrowhead marks the position of the thalamic staining visible at this level. Detailed examination at higher magnification, and β -galactosidase histochemistry on cryostat sections (not shown) confirmed that the X-gal reaction product in thalamus and cortex, like that in the retina (Fig. 2), was present in neurons.

3.4. Expression of endogenous A3 adenosine receptor in cerebrospinal vasculature and retina

To date, there have been no reports of A3 adenosine receptor expression in the cerebrospinal vasculature or retina. In light of observed β -galactosidase activity in these tissues in pSA3Z mice, we sought to determine if endogenous A3 adenosine receptor message could be detected in these tissues. In situ hybridization of mouse retina and cerebrospinal vasculature, using a probe derived from the second exon of the mouse A3 adenosine receptor gene, did not yield specific signal after 3 weeks of exposure (data not shown). Low levels of expression would likely preclude detection of signal by in situ hybridization, which is a far less sensitive method of detection when compared with the assay for β -galactosidase activity. Hence, we turned to a more sensitive method for examining these tissues, involving RT-PCR. RNA was isolated from several organs, including retina and cerebrospinal vasculature, of several Sprague–Dawley rats. Subsequent RT-PCR amplification using primers specific for rat A3 adenosine receptor cDNA showed significant levels of A3 adenosine receptor message in both retina and cerebrospinal vasculature, in contrast to a very low level of message in other tissues, such as skeletal muscle. GAPDH message was uniformly high in all organs examined (Fig. 4).

3.5. β -Galactosidase activity in brains of transgenic mice

Brain A3 adenosine receptor expression has been described in several species including human, sheep, rat, and mouse [1]. Sites of expression of the A3 adenosine receptor within the brain have been debated. In a previous study involving in situ hybridization, we reported A3 adenosine receptor expression in the thalamus and hypothalamus of the mouse brain, but not in the cortex and cerebellum [19]. We were interested in determining the profile of β -galactosidase activity in the brains of the pSA3Z mice. To this end, brains were dissected, sliced sagittally and coronally, and stained for β -galactosidase activity (Fig. 5). Specific staining was detected in brain in all four transgenic lines. Regions within the thalamus and hypothalamus demonstrated specific neuronal staining in more than one transgenic line (#26 > #5 > #29 = #30). Although the specific localization in these areas, and the intensity of staining, was not uniform among the transgenic lines, staining was consistently present or strong in three of the lines (#26, #5, #29) in anterior and lateral nuclei of the thalamus, with particular prominence in the lateral geniculate. In the cerebral cortex, scattered large neurons in deeper layers exhibited β -galactosidase activity, and the cerebellum displayed staining concentrated in its lateral margins. In general, the cortical staining was significantly stronger in the #26 line, weaker or absent in the other lines. In the brainstem, β -galactosidase staining was present in two lines in the interpeduncular nucleus, and in the associated habenulo-interpeduncular tract (#26 > #5); three lines showed scattered staining of neurons in the superior colliculus (#5 > #26 > #29).

4. Discussion

We have examined the in vivo promoter activity of a 2.3 kb fragment of murine genomic DNA located 5' upstream of the A3 adenosine receptor coding region by coupling it to the *E. coli lacZ* coding region. The profile of β -galactosidase activity in transgenic mice generated using this construct was

examined, and locations of specific β -galactosidase activity were compared with the pattern of expression of the endogenous A3 adenosine receptor gene. Four major sites of β -galactosidase activity – testis, brain, retina, and cerebrospinal vasculature – were detected, and analyzed in detail. Two of these sites, the retina and cerebrospinal vasculature, have not been previously described as locations of A3 adenosine receptor expression.

We demonstrated that the profile of β -galactosidase activity in our transgenic mice is consistent with that described in previous reports for A3 adenosine receptor mRNA expression in the testis of the rat. Furthermore, we have demonstrated by in situ hybridization that the pattern of testicular expression of the endogenous mouse A3 adenosine receptor gene is consistent with both sets of observations. In the brain, endogenous A3 adenosine receptor and β -galactosidase activity were detected in the thalamus and hypothalamus. These results demonstrate that the 2.3 kb promoter used in our studies can direct transcription in vivo in a tissue- and cell-specific manner.

We have also demonstrated specific β -galactosidase activity driven by the murine A3 promoter in sites not previously connected with endogenous A3 adenosine receptor expression, specifically in the retina. The consistency of this histochemical activity in multiple, independent transgenic lines indicates that the retinal β -galactosidase expression is not an artifact of transgene insertion site but is instead an accurate reflection of the capacity of the 2.3 kb A3 promoter region to direct tissue-specific transcription. Our RT-PCR analysis suggests that the activity of this promoter region in the transgene may be matched by significant expression of the endogenous A3 adenosine receptor gene in the rodent retina. A potential role for the A3 adenosine receptor in the retina has yet to be explored, but there are several reports describing modulation of axonal regeneration in cultured retinal ganglion cells by different nucleosides, including guanosine, adenosine, and inosine [20–22]. Conversely, the scattered cerebral cortical staining observed in different transgenic lines and the cerebellar staining seen in line #26 were not consistent and could be the result of a low level of transgene activation dependent upon the different sequences surrounding each (random) genomic insertion point in each of the transgenic lines. Nevertheless, the overall consistency of transgene activity in thalamic neurons, together with the match of specific β -galactosidase localization in retinal ganglion cells of three lines, demonstrates that the A3 promoter region in our transgenic construct contains elements that direct expression to specific groups of neurons.

As with the retina, we have shown a potential match of transgene activity in different lines with endogenous A3 adenosine receptor expression in smooth muscle cells in blood vessels around the brain and spinal cord. Our observations indicated clearly, however, that only a subset of all VSMC in the cerebrospinal vasculature exhibited β -galactosidase activity. This might indicate a mosaic pattern of activation of the A3 adenosine receptor gene promoter within the vasculature, as observed for the smooth muscle myosin heavy chain promoter in transgenic mice [23]. In that case, the promoter region examined was active only in some VSMC in vivo, suggesting that a larger promoter fragment or an intronic sequence might direct high expression in other VSMC. Some reports have described significant transcriptional heterogene-

ity in VSMC [24,25]. A more detailed analysis will be necessary to identify all of the sequences in the vicinity of the A3 adenosine receptor gene that modulate smooth muscle expression.

It is established that the endogenous A3 adenosine receptor gene is active in cells of the myeloid lineage, including mast cells and macrophages [5,26]; cultures of bone marrow-derived mast cells show high levels of A3 expression [5]. We have been unable to detect β -galactosidase activity in peritoneal mast cells and lung tissue of pSA3Z transgenic mice by histochemistry (data not shown). RT-PCR analysis of cultured bone marrow-derived mast cells and lung tissue has also failed to reveal any specific expression of *lacZ* mRNA in our transgenic pSA3Z mice. It is possible that the sensitivity of the methods used may be insufficient to detect low levels of A3 promoter activity in these locations. An alternative explanation for this observed discrepancy is that sequences not included in the 2.3 kb fragment used in our construct are necessary to direct myeloid-specific A3 adenosine receptor expression. It is plausible that sequence elements that are either more distant from known transcriptional start sites, or within introns [27,28], could be required to endow a transgene with the full transcriptional capacity of the endogenous A3 adenosine receptor gene.

In summary, our transgene faithfully recapitulates cell-specific expression in testis, and also directs expression to neurons in the thalamus in the brain, to ganglion cells in the retina, and to smooth muscle cells in the cerebrospinal vasculature. We have shown that the latter two sites do, in fact, express significant levels of endogenous A3 adenosine receptor mRNA. Lines #5 and #26 could serve as convenient models for studying, in vivo, the effect of different ligands on the suppression or enhancement of transcriptional activity of the A3 adenosine receptor gene in the brain, testis, retina, and cerebrospinal vasculature. In addition, the promoter region we have characterized may be used to drive the tissue-specific expression of foreign genes in areas already expressing the endogenous A3 adenosine receptor, in order to examine potential coregulation.

Acknowledgements: We thank Dr. Herbert Kagan for valuable insight, and Hou Xiang Xie and Robin McDonald for transgenic core services. This work was supported by Grant HL13262-NHLBI. R.Y. was supported by NIH Institutional Training Grant #T32 HL 07035-26, 'Biology of the Lung, A Multidisciplinary Approach', from the National Heart, Lung and Blood Institute.

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